Growth and Purification of PGK-2 Protein

Including a Study on Extraction of PGK

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Introduction:

Rodents, among other pests, have been rather intimately tied to mankind for millennia. While many rodents certainly play a valuable role in their respective environments, various species are capable of wreaking havoc on their eco-systems and can be quite harmful and financially damaging, especially to humans. Three major reasons rats and other rodents are so detrimental is that they consume and/or damage human foods by spoiling them with urine and droppings, their nature of gnawing and burrowing causes damage to possessions as well as structural instabilities, and they are very effective at quickly transmitting and propagating disease. The last reason has historically been the most notorious, with such examples as the financial and human loss caused by the Black Plague. The bubonic plague or "Black Death" killed nearly a third of the population in Europe (some 25 million people) in the mid 1300's, less than five years after the disease arrived in Europe from China.[1]

While only 20 or so of the 1700 species of rodents found worldwide are considered significant pests, the economic impact of these few species cannot be ignored. Rodents are prolific breeders and can have up to five litters per year under optimal conditions, so their reproduction and spread can occur quickly. In fact, the house mouse (*mus musculus*) is now the most widespread mammal in the world.[1] They are capable of eating 10% of their own body weight in one day, so the damage they inflict can be substantial. Net crop losses resulting from damage done by rodents each year could feed up to 200 million people, which is comparable to the population of Indonesia. With so much worldwide focus on reducing world hunger and poverty, the damage caused by

rodents cannot be overlooked and rodent populations must be managed more successfully.

Benjamin Franklin's maxim, "an ounce of prevention is worth a pound of cure," is equally true for rodents as it is for other pests and diseases. Control strategies for rodent infestations should aim at preventing losses and thus requires a pro-active mindset instead of the normal reactive approach. Therefore, although not always practical the ideal cornerstone of any rodent control campaign should be to create environmental conditions which will discourage or prevent the pests from populating or remaining in a targeted area. Sanitation and proofing are two effective methods of preventing the infestation of rodents in smaller areas, but more effective means are needed for larger areas.

Another technique of rodent control involves mechanical means. Mechanical means of rodent population control have been used by man for quite some time and are still common today, usually involving directly trapping pests. Sticky or glue traps are another way of catching rats and mice, but these are usually quite uncomfortable for the animal and so are considered inhumane. While some manufacturers tout the effectiveness of ultrasonic devices as rodent repellents, there is no research to confirm this. In fact, some experts believe rodents can become habituated to the sounds so that they are no longer bothered by them, or they hide in "sound shadows" (around objects that block the sound) and avoid being repelled. Although generally effective for households and small businesses, mechanical means of rodent control are not practical for warehouses and other large areas.

Chemical methods are another alternative for rodent control. Anticoagulant rodenticides are often used and act by interfering with the blood clotting mechanism of the body.[2] This leads to the gradual death of the animal because of blood loss through both external and internal hemorrhaging. It is normal for an animal to experience minute internal wounds (such as the breaking of small capillaries) caused by normal motion. However, without the ability to clot these capillaries gradual blood loss occurs. The rodent experiences almost no pain, feeling more and more tired until death.[2]

Resistance to some anticoagulants has already been observed in some developed countries, where they have been used very extensively over long periods. Resistance sometimes occurs as a result of animals ingesting non-lethal doses, sometimes caused by improper application. So far, resistance has not been of serious importance in tropical countries, partially because new compounds (such as difenacoum, brodifacoum, bromadiolone) are now available in most countries.[2]

While the fumigation of crops and buildings for insect control can lead to the incidental death of rodents, fumigation can also be done with rodent control as the primary goal. Although fumigation can be very effective, it can also be quite expensive and dangerous. It involves applying poison gas directly into the rodent burrows, leading to death of all individuals therein. The gas can be generated by the contact of powder or tablets with moisture in the soil. Methyl bromide gas can otherwise be pumped into the burrow system.

While methods described above such as poison baits and fumigation can be successful, these approaches are often not cost effective and can be harmful to other unintended animals as well as humans. A more feasible and environmentally friendly

method of controlling rodent populations is undoubtedly needed. The method of immuno-contraception has the potential to safely control specific species populations while still remaining cost effective.

The technique of immuno-contraception relies on an animal creating antibodies to components of its own reproductive system. The animal's own immune system acts to render the animal infertile, allowing for rodent population control. Introduction of an antigen that targets sperm in particular leads to an immune response and antibodies against the sperm. A loss of fertility results in both male and female pests as an accumulation of antibodies occurs. One such antigen that can lead to infertility in mice is phoshpoglycerate kinase-2, or PGK-2.

The mammalian phosphoglycerate kinase (PGK) gene family includes two functional loci. One is the ubiquitously expressed and intron containing X-linked PGK-1 gene and the other is the intron-less PGK-2 gene expressed only in spermatogenic cells.[3] The PGK-2 gene is an intron-free functional gene that possesses all of the characters of a processed gene including a full open reading frame and the remnants of a poly (A) tail.[4] PGK-2 is a glycolytic enzyme involved in energy production. More specifically, PGK-2 catalyzes the energy producing reaction of 1, 3-diphosphoglycerate and ADP to 3-phosphoglycerate and one molecule of ATP. The expression of mouse PGK-2 is activated during the spermatogenic pathway at the pachytene spermatocyte stage. In addition, PGK-2 is specific to the testis and is needed for sperm movement.[5] In acting as an antibody, PGK-2 renders sperm from the male incapable of reaching eggs in the female by possibly inhibiting the acrosomal reaction or causing an accumulation of sperm within

the oviduct. It is also possible that antibodies are moved into the zygote by the sperm, causing egg development to stall.[6]

The objective of the work described herein was to grow and purify PGK-2 protein so that further trials on mice could be conducted. Previous experimental trials have led to a 30% reduction in mice fertility, while the desired fertility reduction is closer to 60%. In addition to growth and purification of PGK-2, a study to optimize sonication of PGK-2 GST cells was conducted and resulted in an update to the previous protocol.

Effects of Sonication on PGK Extraction:

This experiment aimed to determine if the amount of sonication that cultured *E-coli* cells undergo effects the amount and activity of PGK extracted. It is possible that using a larger amount of sonication will allow for extraction of more PGK, or PGK of higher activity. However, over sonication is also possible and would result in extraction of PGK of lower activity (possibly due to denaturation or enzyme damage). These adverse effects are highly undesirable and are to be avoided.

This experiment involved sonicating three samples for three different levels of sonication, with the activities of each of these samples being assayed for comparison. The amount of sonication used for the sample that yielded the highest activity would be considered the optimal sonication amount. The conclusions of this study are outlined below in the Results section.

Procedures and Materials:

Existing PGK-2 GST cells were used to further express and collect unpurified PGK-2 GST. A glutathione-agarose column was then used for protein fusion (PGK-2 GST). It was necessary to then cleave the fusion protein directly from the agarose beads, which was done using thrombin cleavage buffer and a shaker over a week-long period. A Sephacryl S-200 column run further separated the PGK-2 from the uncleaved GST-PGK2. Protein isolation and purification were examined using PGK-2 assays, BCA protein assays, and SDS-PAGE gel runs.

Growing PGK-2 GST:

1. Prepared 500mls LB agar (using 18.5g agar and de-ionized water to 500mls)
2. Autoclaved for 25 minutes, then cooled and added 835µls of 60mg/ml Ampicillin
3. Poured 3 Petri dishes and let harden, then put in 37C incubator overnight
4. Used PGK-2 GST cells to streak plates
5. Made up 3 50ml flasks (250mls) of LB broth (25g/L)
6. Autoclave for 20 min., let cool then add 83.5λl/50mls Ampicillin
7. Added single colony from plate using sterilized toothpick to each 50mls broth
8. Put in shaker at 37C for overnight growth
9. Autoclaved 3 1500ml batches of LB broth in 4L flasks
10. When cool added 100mg/1L of Ampicillin to each flask
11. Inoculated each 1500ml flask with the 50mls of cells grown overnight
12. Put each 4L flask into the incubator-shaker, checking for the OD 600nm reading to pass 0.6 (required 2hrs and 30 minutes)

13. Added IPTG (0.5mM = 179mg/1.5L) to each 4L flask to induce cells for rapid growth.
14. Grew 4 to 6 hrs (5 hrs)
15. Centrifuged cells at 5000 RPM for 15 min. to separate. Poured off the supernatant and stored the cells in Freezer #1.
16. Three tubes were collected with a total mass of 13.12 g

Purification of GST-PGK2

1. Cells grown up and frozen (approximately 4-5 g) should first be resuspended in 25mls of lysis buffer in a 50ml tube (or appropriate container for sonication). HEPES buffer (50mM HEPES, pH 7.5, 50mM NaCl)
2. Sonication is done using the new sonicator located under the hood. The smaller needle point should be used. After turning the sonicator on, make sure to answer NO when asked if you are using a Micro Tip.
3. Sonication of the tube of cells is then done six times for 30 second pulses, with 30 seconds to 1 minute breaks in between. The tube can be placed on ice during these breaks to ensure the temperature is not elevated too much.
4. The solution can then be centrifuged in the Beckman GS-15R centrifuge using the F1010 rotor at 15,000rpm for 30min to 1 hr.
5. Filter with syringe. 45mls total

Glutathione (GST) Column Chromatography and Thrombin Cleavage

1. Prepare a 5ml glutathione-agarose column. Approximately .5g glutathione-agarose is swollen in 200mls ddH_2O for 1-2 hrs at room temp
2. Put lysate into 5mls of glutathione-agarose gel in 50ml blue top tube

3. Let shake for 1hr on shaker in old room
4. Pour into column and run off, collecting flow through with several (2-5 mls) of lysis buffer
5. To increase purity, wash the column with 100mls lysis buffer with 1% Triton X-100
6. Wash the column with 1L lysis buffer (overnight wash) to remove Triton X-100 and extraneous protein
7. Resuspend the glutathione agarose in lysis buffer to create a total volume of 13.5ml and transfer the suspension to a 15ml tube. This is where cleavage of the fusion protein from the agarose beads begins
8. Add 0.5ml thrombin cleavage buffer and 50 units thrombin protease
9. Place the tube on a shaker and slowly mix the suspension overnight in cold room. At least half of the PGK-2 GST should be cleaved after four days. More thrombin should be added as needed (depending on results of SDS-PAGE)
10. Capture the cleaved PGK-2 by transferring the 15ml suspension to an empty column, then elute the supernatant from the beads and capture the flow through
11. Add 35ml lysis buffer to wash all of the cleaved PGK-2 from the column. The GST remains on the column. More thrombin can be added to cleave some of the remaining PGK-2 (Added 50λ of additional thrombin)
12. The 50ml volume of cleaved PGK-2 can be concentrated by using Amicon 10,000 MWCO membranes (concentrated to 2mls)
13. Run Sephacryl S-200 column to separate the PGK-2 from the uncleaved GST-PGK-2

14. Column was 43cm x 1.5cm, 34mls of gel
15. Poured column and equilibrated with GST lysis buffer
16. The 2mls of previously concentrated PGK-2 was further concentrated to 1ml (10λ of concentrate saved to run activity assay and do BCA Protein Assay)
17. Loaded 1ml of concentrated PGK-2 (with GST- PGK-2) on column. Ran buffer to top of gel before loading. Ran the sample into the gel (layer buffer on the top and run it into gel)
18. Collect 1ml fractions and do OD280's using Spectrophotometer on each tube (Tubes 27-30 and 37-40 are to be combined and concentrated as pure PGK-2)
19. Storage of PGK-2 at -70C to -80C should maintain complete functionality

BCA Protein Assay (Analysis of amount of PGK-2 present)

1. Made 1mg/ml BSA for Standard Curve (10mg BSA + 10mls DH_2O)
2. Standard curve dilutions: 1mg/ml, 0.8 mg/ml, 0.6 mg/ml, 0.4 mg/ml, 0.2 mg/ml, 0.1 mg/ml using PBS for dilutions
3. Mixed 5mls of Reagent A and 0.01mls of Reagent B to make Working Reagent
4. Mark well plate with regions for standards, blanks, and protein sample
5. Test was performed by pipetting 10λ of each STD and protein sample into a well (duplicated performed for higher accuracy)
6. Add 200λ of Working Reagent to each well
7. Incubate the well plate at 37C for 30 min
8. Read on plate reader at 600nm

PGK Protein Assay

1. Reagents A through H were made fresh before the assay

2. 470λ Reagent A, 33.3λ Reagent B (GAP), 16.7λ Reagent C (B-NAD), 16.7λ Reagent D (ADP), 83.3λ Reagent E ($MgSO_4$), 333λ Reagent F (Glycine), and 16.7λ Reagent G (GAPDH) were pipetted into a cuvette
3. The cuvettes were mixed by inversion and allowed to sit for 15min to equilibrate
4. The spectrophotometer was set to 340nm to measure the absorbance.
5. After zeroing each cuvette, 33.3λ of sample was added, the cuvette was mixed by inversion, and the absorbance measured for 5 min.
6. The rate of change (A340nm/time) allows for determination of PGK-2 amounts

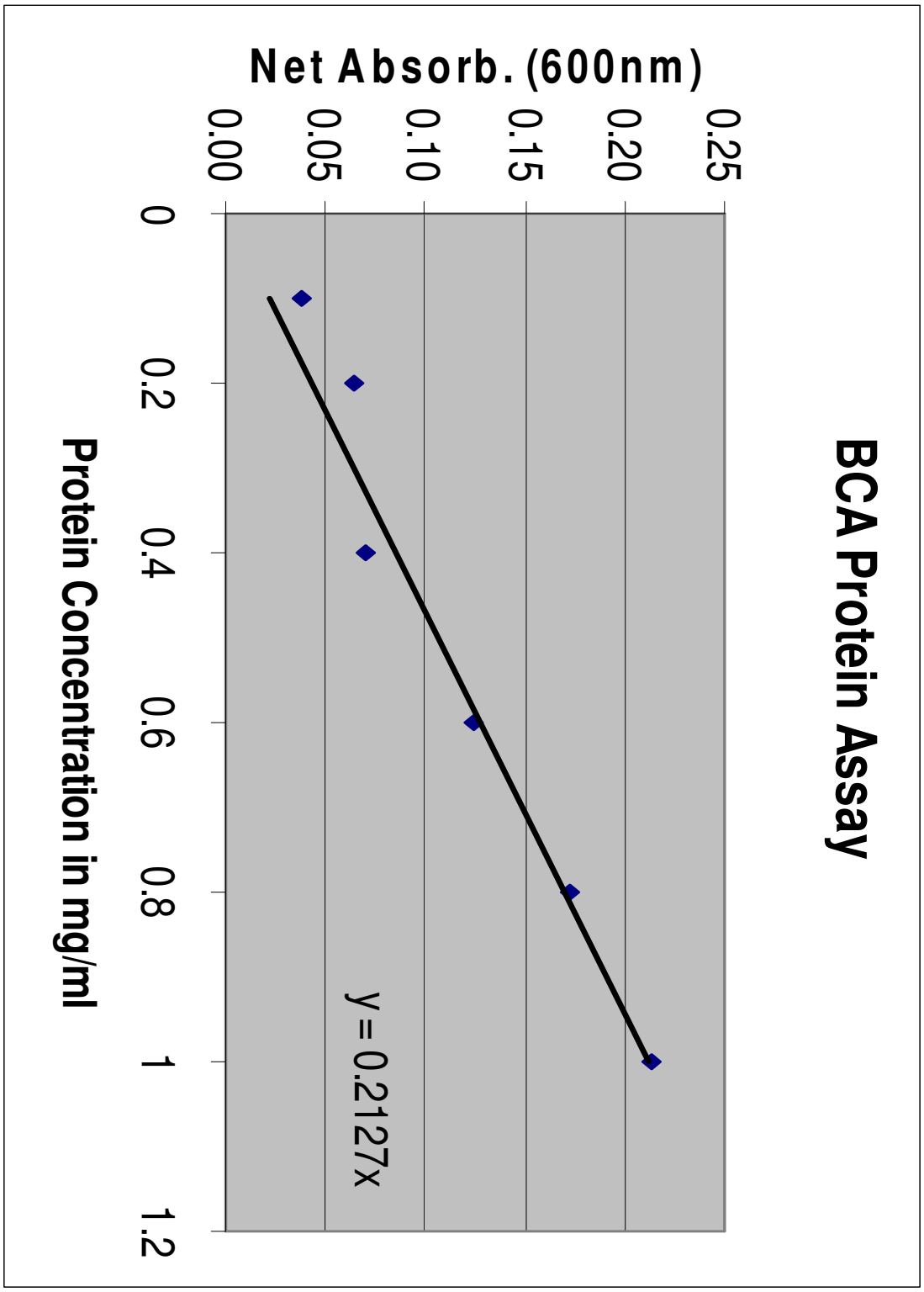
Study: Effects of Sonication on PGK Extraction

1. Three samples were used, each including 0.6g of pelleted PGK-2 GST cells and 4 ml lysis buffer (HEPES).
2. The three samples underwent 3, 6, or 9 thirty second pulses of sonication, with 30 seconds to 1 minute breaks in between.
3. The 4 ml sample plastic centrifuge tubes were placed in a Beckman GS-15R centrifuge using the F1010 rotor at 15,000 rpm for 30 mins. When removed, there was a slight color (yellowish) to the supernatant, so 15 more minutes of centrifugation were used.
4. A PGK Assay was run for the three samples to measure activity.

Data, Results and Conclusion:

Data collected from the BCA protein test and PGK-2 Assay can be used to calculate an assortment of information. The data collected from the PGK-2 Assays on GST purified PGK-2 in Tubes 37-40 and 27-30, as well as the Filtrate from Tubes 27-30 and 37-40 were recorded in the form ΔA_{340}/time. The average over the five minutes of recordings was taken, multiplied by the dilution factor (200 for pure PGK-2, 10 for Filtrate), and then multiplied by the sample volume to give the total Enzyme Units.

The readings for the blanks on the BCA were averaged to give one calibrating value, which is subtracted from all sample readings (including the standard values). A standard curve was drawn from the standard values and a line of best fit assigned (See *BCA Protein Assay* below and attached *BCA Protein Assay Calculations and Values*). Ideally, this line would fit the points perfectly and pass through the origin. Absorbance readings for PGK-2 Tubes 27-30 and 37-40 and Filtrate Tubes 27-30 and 37-40 were averaged for each subset and used in conjunction with the line of best fit to determine the amount of mg/ml of protein present per sample (the protein concentration). When multiplied by the total volume this yields the amount of total protein. Each sample's specific activity was calculated by dividing the total Enzyme Units by the total amount of protein.



A table summarizing all of the calculations and findings is provided below.

PGK-2 Purification Table

Sample	Volume	Total Protein	EnzU/ml	Total EnzU	**Specific Activity**
PGK-2 from Tubes 37-40	1.0 ml	2.28 mg	22.0 EnzU/ml	22.0 EnzU	9.65 EnzU/mg
Filtrate from Tubes 37-40	3.0 ml	1.23 mg	1.14 EnzU/ml	3.42 EnzU	2.78 EnzU/mg
PGK-2 from Tubes 27-30	0.7 ml	0.826 mg	20.4 EnzU/ml	14.28 EnzU	17.29 EnzU/mg
Filtrate from Tubes 27-30	3.0 ml	0.613 mg	1.8 EnzU/ml	5.4 EnzU	8.81 EnzU/mg
Average/Sum of 11/2006 Runs (Tubes 27-30 & 37-40)	7.7 ml	4.95 mg	5.86 EnzU/ml	45.1 EnzU	9.11 EnzU/mg
MM, JL 11/06 Run, Pure PGK-2	1.7ml	3.106 mg	21.2 EnzU/ml	36.04 EnzU	11.6 EnzU/mg
Greg 11/05 Run	4.2 ml	5.04 mg	241.0 EnzU/ml	1012 EnzU	200.8 EnzU/mg
JL 12/05 Run	3.0 ml	3.5 mg	282.5 EnzU/ml	847.5 EnzU	242.1 EnzU/mg

The total enzyme units and specific activities of the isolated pure PGK-2 were lower than expected, as was the total amount of protein when compared to past trials. It is probable that some of the experimental adjustments resulted in a loss of some of the PGK-2 and especially in the loss of activity. It was recognized that more thrombin

cleavage buffer (approximately 2x) should initially have been added to better cleave the PGK-2 GST complex. This is because at least some of the PGK-2 GST fusion protein had not separated, even after a one week period and some additional thrombin buffer was added. Also, there is no known harm from using excess thrombin to initially cleave as much PGK-2 as possible. It is also likely the amount of processing incurred as a result of poor cleavage (especially the S-200 column) resulted in more PGK-2 being lost in the columns and washes, and the collected PGK-2 becoming less active. When repeated in the future, a larger initial thrombin cleavage buffer should hopefully bypass the need to run the fusion protein through additional separation columns and lead to more total protein isolation and higher activity.

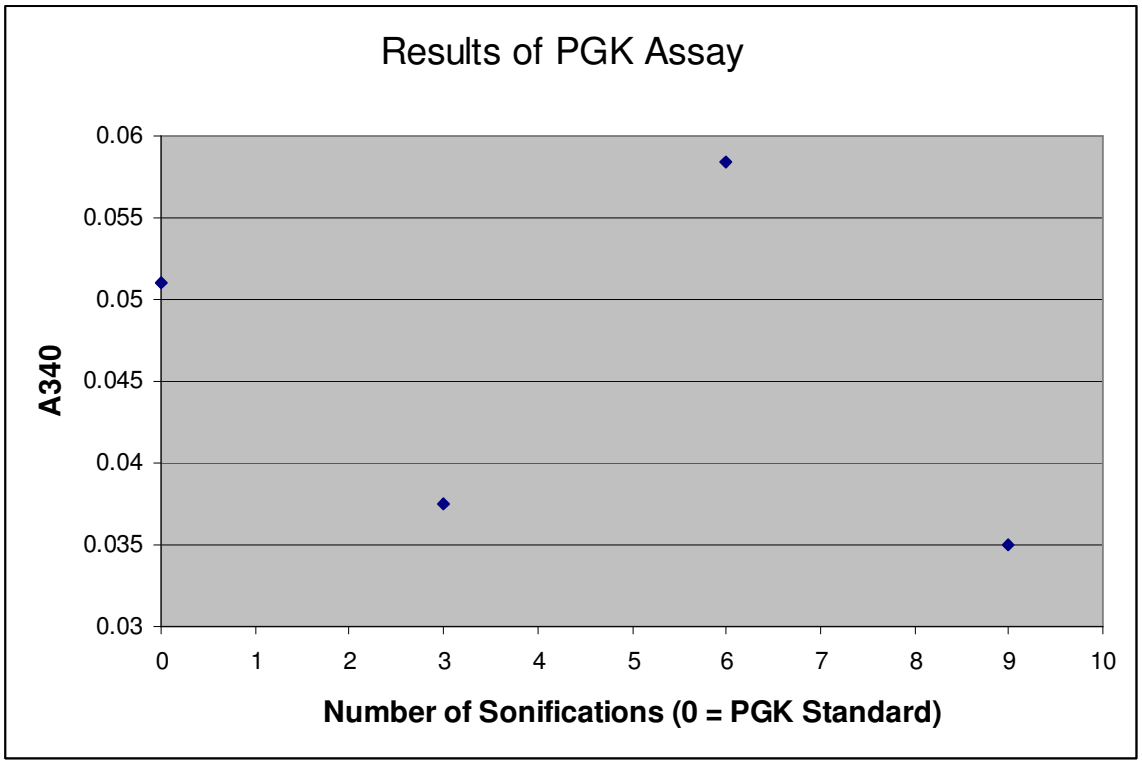
Results of *Effects of Sonication on PGK Extraction*

For smaller sample sizes, such as the 4 ml samples used here, sonicating for pulses of 10 seconds instead of 30 seconds is crucial. Thirty second pulses had been attempted initially for the 4ml samples but this resulted in over heating as well as excessive foaming. A PGK assay was conducted on the three samples. From the assay we found an average A_{340} of 0.051 for the PGK standard solution. The sample sonicated 3 times yielded an average A_{340} of 0.0375, and the sample sonicated 9 times yielded an average A_{340} of 0.035. The sample sonicated six times had the highest activity of all, with an A_{340} of 0.0584.

Average A_{340} of PGK Assay Samples

Sample	Average A_{340}
PGK Standard	0.051
3 Sonications	0.0375
6 Sonicatons	0.0584
9 Sonicatons	0.035

This data was translated into a graph for visual comparison:



Experiments conducted in the future may optimize the extraction of PGK by using six pulsed sonications. Obviously, sample size should be considered when determining how long these pulses should last. A small sample size (4 mls) was used here and so pulses were only done for ten seconds because adverse effects such as over heating and foaming were observed when thirty second pulses were attempted. Larger volumes, however, such as the 25ml sample normally employed necessitate the use of thirty second pulses to ensure complete sonication. While it is not entirely accurate to extrapolate the results obtained here using 4 ml samples and 10 second pulses to experiments requiring 25ml samples and thirty second pulses, the range of discrepancy can be minimized, thus allowing for general acceptance. Most significant to our assumption that our small scale conclusions can be applied to the larger scale is the control of heat. Excessive heating can lead to enzyme denaturation and loss of activity. While this has not been observed in past 25 ml runs, it can be accounted for and prevented by keeping the sample on ice throughout sonication. It should also be noted that all future PGK sonications should be conducted under the hood. This prevents aerolized *E-coli* from escaping the tube and possibly being ingested. While this particular strain of *E-coli* is not particularly dangerous, this is a worthwhile precaution.

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References:

1. Rodents: a Gnawing Problem. New Agriculturist, on-line @ http://www.new-agri.co.uk , accessed 05/2007.
2. Grain Storage Techniques – Evolution and Trends in Developing Countries. Food and Agriculture Organization of the UN, Corporate Document Repository. Accessed 05/2007 @ http://www.fao.org/documents.
3. McCarrey, John R. Molecular Evolution of the Human PGK-2 Retroposon. *Nucleic Acids Research*. 1990 February 25; 18(4): 949–955.
4. McCarrey J. R. & Thomas K. (1987) Nature 326, 501– 5.
5. Goto, M, Masamune, Y, and Nakanishi, Y. A Factor Stimulating Transcription of the Testis-specific Pgk-2 Gene Recognizes a Sequence Similar to the Binding Site for a Transcription Inhibitor of the Somatic-type Pgk-1 Gene. *Nucleic Acids Res*. 1993 January 25; 21(2): 209–214.
6. Hirshhorn, Daniel. Dissertation: Development of Contraceptive Vaccines for the Control of Rodents and other Mammals. (2003).